Early Alcoholic Liver Injury: Formation of Protein Adducts with Acetaldehyde and Lipid Peroxidation Products, and Expression of *CYP2E1* and *CYP3A*

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The formation of protein adducts with reactive aldehydes resulting from ethanol metabolism and lipid peroxidation has been suggested to play a role in the pathogenesis of alcoholic liver injury. To gain further insight on the contribution of such aldehydes in alcoholic liver disease, we have compared the appearance of acetaldehyde, malondialdehyde, and 4-hydroxynonenal adducts with the expression of cytochrome P-450IIE1, and cytochrome P-4503A enzymes in the liver of rats fed alcohol with a high-fat diet for 2 to 4 weeks according to the Tsukamoto-French procedure and in control rats (high-fat liquid diet or no treatment). Urine alcohol and serum aminotransferase levels were recorded, and the liver pathology was scored from 0 to 10 according to the presence of steatosis, inflammation, necrosis, and fibrosis. The ethanol treatment resulted in the accumulation of fat, mild necrosis and inflammation, and a mean liver pathology score of 3 (range: 1 to 5). Liver specimens from the ethanol-fed animals with early alcohol-induced liver injury were found to contain perivenular, hepatocellular acetaldehyde adducts. Malondialdehyde and 4-hydroxynonenal adducts were also present showing a more diffuse staining pattern with occasional sinusoidal reactions. In the control animals, a faint positive reaction for the hydroxynonenal adduct occurred in some of the animals fed the high fat diet, whereas no specific staining was observed in the livers from the animals receiving no treatment. Expression of both CYP2E1 and CYP3A correlated with the amount of protein adducts in the liver of alcohol-treated rats. Distinct *CYP2E1*-positive immunohistochemistry was seen in 3 of 7 of the ethanol-fed animals. In 5 of 7 of the ethanol-fed animals, the staining intensities for *CYP3A* markedly exceeded those obtained from the controls. The present findings indicate that acetaldehyde and lipid peroxidation-derived adducts are generated in the early phase of alcohol-induced liver disease. The formation of protein adducts appears to be accompanied by induction of both *CYP2E1* and *CYP3A*.

Key Words: Ethanol Metabolism, Lipid Peroxidation, Liver Disease.

TOXIC EFFECTS of acetaldehyde (AA), the first metabolite of ethanol, and aldehydic products of lipid peroxidation have been suggested to play a significant role in the pathogenesis of alcohol-induced liver damage.[1-4] A growing body of evidence supports the idea that several types of deleterious aldehyde-derived protein modifications are formed in vivo as a result of alcohol consumption.[5-12] Recent studies in ethanol-treated micropigs, which exhibit a wide spectrum of histopathological features of alcoholic liver disease, have indicated that the formation of aldehydic products in the liver coincide with elevated serum transaminases and progressive histopathology.[13] However, in rats treated with ethanol in conventional pair-feeding procedures, no significant liver pathology and fibrosis usually occurs, and very few AA and malondialdehyde (MDA) adducts were found even after several weeks of ethanol treatment.[9]

Recently, the establishment of a continuous in vivo enteral feeding protocol has provided a new tool for examining the effects of ethanol in the liver.[3,14] Experiments with this model have demonstrated the production of most of the typical histopathological features of alcoholic liver disease as a result of ethanol feeding together with a high-fat (corn oil) diet.[3,14] The histological alterations in the liver observed in this model also resemble those occurring in human alcoholics with early stages of liver injury.[3,14,15] Recent studies in this model have suggested a joint occurrence of enhanced lipid peroxidation and induction of cytochrome P-450IIE1 (CYP2E1).[16-18] Furthermore, it was recently demonstrated that MDA and 4-hydroxynonenal (4-HNE) protein adducts are formed in high levels, together with a significant aggravation of liver injury and fibrogenesis when administered together with iron, an enhancer of oxidant stress.[19] However, the question whether or not AA and lipid peroxidation-derived protein adducts are formed with ethanol-containing high-fat diet without iron supplementation has not been examined. Furthermore, no comparisons between cytochrome P-450 induction and the generation of various reactive aldehydic products have been available.

The present work was designed to examine whether rats receiving a high-fat diet, together with ethanol, produce hepatic aldehydic products derived from lipid peroxidation and ethanol metabolism in the liver in the early phase of liver disease. The appearance of such products was compared with serum aminotransferase markers of liver disease, blood ethanol levels, and histopathological alterations for each animal. The cellular distribution of protein adducts in the liver were also compared with that of ethanol-

PM3006739248

inducible CYP2E1 enzyme. Because recent cell culture experiments[20] have also indicated the induction of cytochrome P-4503A (CYP3A) by ethanol, we extended the present studies to examine the immunohistochemical localization of this cytochrome.

MATERIALS AND METHODS

Animal Model

The present animal protocol has been previously described in detail.[15,21] Male Wistar rats weighing ~300 g were used. The animals were housed in an AAALAC-approved facility with lights on between 6 AM and 6 PM. They were implanted with long-term intragastric cannula to enable continuous intragastric infusion of the high fat diet (37% of calories as corn oil). It contained 23% protein, 5% carbohydrate, and minerals, vitamins, and ethanol or isocaloric maltose dextrin (35%). All of the experiments described in the present work were conducted in compliance with institutional guidelines and were in full compliance with the Public Health Service *Guide for the Care and Use of Laboratory Animals.*

Liver Histology

Rats underwent biopsy and autopsy after 2, 3, or 4 weeks of ethanol exposure. Liver tissues were fixed in a 10% formalin solution, embedded in paraffin, and stained with hematoxylin and eosin to assess inflammation and necrosis. Liver pathology was scored blind to the knowledge of the immunohistochemistry data using the system described by Nanji and coworkers,[22] in essence as follows—steatosis (the percentage of liver cells containing fat): <25%, 1+; 25–<50%, 2+; 50–<75%, 3+; and >75%, 4+. Inflammation and necrosis were scored as 1 focus per low-power field (1+) and 2 or more foci as 2+. One point was given for each grade of severity of histological abnormality, and a total score was calculated for each liver.

Blood and Urine Collection, Ethanol, and AST Assays

Concentrations of ethanol in urine which, as previously demonstrated,[23] also reflect blood alcohol concentrations, were measured. Rats were housed in metabolic cages that separated urine from feces. Urine was collected over a 24-hr period for each rat in bottles containing mineral oil to prevent evaporation. Each day at 9 AM, urine collection bottles were changed and a 1-ml sample was stored at −20°C in a microtube until analysis. Ethanol concentration was measured by absorbance at 360 nm resulting from the reduction of oxidized NAD by alcohol dehydrogenase. For the determination of the AST levels, blood was collected via the tail vein once a week and centrifuged. Serum was stored at −20°C in a microtube until assay using standard enzymatic procedures.

Preparation of Antisera

Antisera against MDA-low-density lipoprotein (LDL) were raised by immunizing male guinea pigs with homologous MDA-LDL prepared as previously described.[24] The priming immunization was an intradermal injection of 150 µg of antigen in Freund's incomplete adjuvant at 14-day intervals. Conjugation of 4-HNE to LDL was conducted under reducing conditions, and polyvalent antisera were generated by immunizing male guinea pigs with homologous 4-HNE-LDL.[24] Antibodies against the AA-modified protein (AA1) were raised in rabbits using bovine serum albumin (BSA) conjugated with 1 mM AA, prepared under reducing conditions, as immunogen.[6,25] For the detection of CYP2E1 and CYP3A, monoclonal CYP2E1 (1-98-1) and CYP3A2 (2-13-1) antibodies were used.[26,27]

Immunohistochemical Procedures

Serial paraffin-embedded sections were used for immunostainings with the immunoperoxidase technique essentially as described previously[6,9,13]: (1) deparaffinization of the tissue sections; (2) pretreatment of the sections with 3% hydrogen peroxide for 5 min, followed by rinsing in phosphate-buffered saline (PBS) for 5 min; (3) pretreatment with cow colostral whey for 40 min to block nonspecific binding, followed by rinsing in PBS; (4) incubation with primary guinea pig (1:500 in 1% BSA-PBS), rabbit (1:200 in 1% BSA-PBS), or mouse monoclonal (1:50 in 1% BSA-PBS) antibodies, followed by washing three times in PBS; (5) retreatment of sections with cow colostral whey for 40 min; (6) incubation for 1 hr with biotinylated anti-guinea-pig (Amersham, UK), anti-rabbit (Dakopatts, Copenhagen, Denmark), or anti-mouse (Dakopatts) antibodies (1:300 in 1% BSA-PBS); (7) specific binding of the antibody was detected using peroxidase-conjugated streptavidin (Dakopatts) (1:600 in PBS); and (8) immunostaining was visualized with a solution containing 9 mg diamino-benzidine tetrahydrochloride, 10 µl H_2O_2, and 15 ml of PBS and the intensities of the reactions for aldehyde-derived epitopes and cytochrome enzymes were scored blind to the knowledge of histological data.

RESULTS

Clinical and Histological Characteristics

The clinical and histological characteristics of the experimental animals are summarized in Table 1. As previously presented, the weights of the animals fed a high-fat liquid control diet or the corresponding ethanol-containing diet were not different.[15,21] AST levels in the serum of ethanol-fed rats increased gradually during ethanol treatment, the mean AST level in the ethanol-fed rats (167 ± 44 units/liter) being significantly higher than the values from the untreated rats or from rats fed a high-fat control diet (98 ± 28 units/liter), but not given ethanol (p <0.01). The urine ethanol levels varied between 80 and 375 mg/dl (Table 1). Examination of liver histology revealed pericentral lipid accumulation from the ethanol-fed rats but not from controls. Mild focal necrosis and inflammation were present in the centrilobular region of livers from the ethanol-fed rats. The hepatic pathological scores of the ethanol-treated rats varied between 1 and 5 (Table 1). No fibrosis was found in the livers of these animals.

AA- and Lipid Peroxidation-Derived Protein Adducts in the Liver

Immunostaining using antibodies specific for AA (Fig. 1), MDA (Fig. 2), and HNE (Fig. 3)-derived protein epitopes revealed distinct positive reactions in the ethanol-fed animals. The intensities of the reactions, which varied somewhat from animal to another, are summarized in Table 1. The positive reaction for the AA-modified epitopes in each of the ethanol-fed animals was restricted to the pericentral region (Fig. 1A). A more widespread distribution of adducts with perivenular predominance was, however, seen in some animals (Fig. 1B). Except for one animal fed the high-fat diet, AA adduct positivity was not detected in controls (Table 1).

The MDA-derived protein epitopes also revealed distinct positive reactions in the ethanol-fed animals, compared with those from the control animals. However, MDA adducts were usually distributed in a more diffuse pattern than the AA adducts (Fig. 2). HNE adducts were found at

Table 1. Clinical and Histological Characteristics and the Summary of the Staining Intensities for the Various Protein Adducts and Cytochrome Enzymes in Alcohol-Treated and Control Rats

Animal no.	Duration of treatment (weeks)	Urine alcohol	AST (units/liter)	Histology score	AA	MDA	HNE	CYP2E1	CYP3A
Ethanol treated									
EtOH1	2	213	214	4	3	3	2	1	6
EtOH2	2	375	195	5	2	1	1	0	4
EtOH3	3	80	140	3	4	2	2	1	5
EtOH4	4	223	201	1	1	2	1	0	3
EtOH5	4	226	131	5	2	3	2	2	6
EtOH6	4	254	96	2	1	0.5	0	0	2
EtOH7	4	112	193	1	1	1	0	0	2
High-fat diet									
HFD1	2	0	130	0	0	0	1	0	1
HFD2	2	0	111	0	1	1	2	0	2
HFD3	3	0	75	0	0	0	1	0	1
HFD4	3	0	58	0	0	1	1	0	2
HFD5	4	0	123	0	0	0	1	0	2
HFD6	4	0	91	0	0	0	0	0	1

Pathology is scored from 0 to 10 according to steatosis (0–4), inflammation (0–2), necrosis (0–2), and fibrosis (0–2), as described in "Materials and Methods." No fibrosis was detected in livers of these rats. Immunohistochemical stainings: 1–2, mild reaction; 3–4, moderate reaction; 5–6, strong reaction. EtOH, ethanol; HFD, high-fat diet.
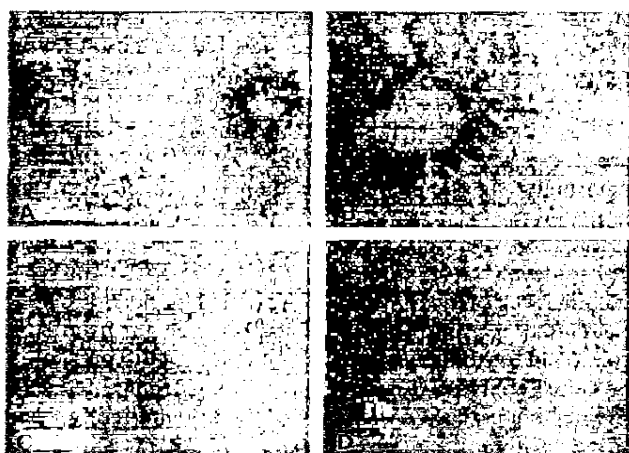

Fig. 1. Immunohistochemical localization of AA-modified epitopes in the liver of rats fed alcohol together with a high-fat diet (A, B). A distinct perivenular localization of AA adducts is seen. Examination of liver histology revealed pericentral lipid accumulation from the ethanol-fed rats, but not from controls. Mild necrosis and inflammation was also present in the centrilobular region of the liver from the ethanol-fed rats. No specific staining for the AA adducts was seen in the liver of animals with the high-fat diet alone (C) nor when the liver of alcohol-fed animals was stained using nonimmune serum (D). (A) Animal no ethanol-1. (B) Animal no ethanol-2. (C) Animal no high-fat diet-1. (D) Animal no ethanol-1. Sites of positive staining are indicated by arrows. Original magnifications, × 250.

Fig. 2. Immunohistochemical localization of MDA-modified epitopes in the liver of rats fed alcohol together with the high-fat diet also revealed positive reactions in the ethanol-fed animals (A, B), compared with those from the control animals fed the high-fat diet (C) or from those with no treatment (D). A perivenular localization of staining is seen with sinusoidal reaction. MDA adducts were usually distributed in a more diffuse pattern than the AA adducts. (A) Animal no ethanol-1. (B) Animal no ethanol-2. (C) Animal no high-fat diet-1. (D) Animal no ethanol-1. Sites of positive staining are indicated by arrows. Original magnifications, × 250.

the same sites and zonal locations as the AA and MDA adducts (Fig. 3). The staining intensities for the HNE adducts correlated significantly with both AA ($r = 0.901, p < 0.01$) and MDA ($r = 0.883, p < 0.01$) epitopes (Table 1).

In the control animals receiving the high-fat diet without ethanol, the stainings for the various protein adducts remained negative except for a diffuse pattern of a faint positive reaction for the HNE adduct in most of the animals fed the high-fat diet (Table 1, Fig. 3C). No specific staining was observed in the livers from the animals receiving no treatment (Fig. 4).
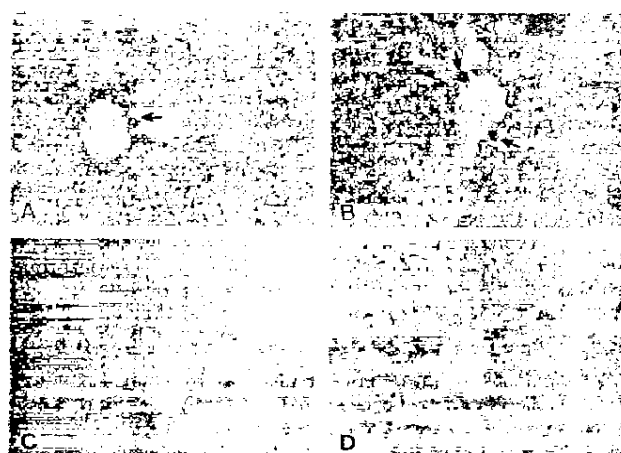
Cytochrome P-450 Enzymes in the Liver

Immunohistochemical stainings for the cytochrome P-450IIE1 enzymes revealed positive staining in zone 3 in three (43%) of the ethanol-fed animals (Table 1, Fig. 5). In four ethanol-fed animals and in all of the control animals, the stainings for CYP2E1 remained negative. In contrast, CYP3A was demonstrated in both the control livers and in the ethanol-fed animals. However, the staining intensities for CYP3A in the centrilobular region were markedly higher in the ethanol-fed animals than in the controls (Fig. 6). Significant positive correlations were observed between the amount of CYP2E1 and MDA ($r = 0.827, p < 0.05$) and HNE ($r = 0.884, p < 0.01$). CYP3A correlated with AA ($r = 0.810, p < 0.05$), MDA (0.888, $p < 0.01$), and HNE ($r =$
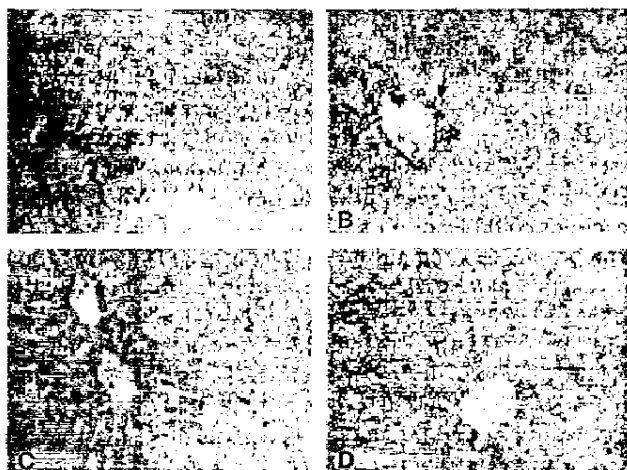
Fig. 3. Immunohistochemical localization of HNE-modified epitopes in the liver of rats fed alcohol together with a high-fat diet (A, B) and in control animals fed the high-fat diet (C) or in animals with no treatment (D). A perivenular staining pattern was seen in the alcohol-fed animals. HNE adducts were found at the same sites and zonal locations as the AA and MDA adducts. Staining intensities for the HNE adducts correlated significantly with both AA ($r = 0.901$, $p < 0.01$) and MDA ($r = 0.883$, $p < 0.01$) epitopes (Table 2). A slight positive reaction for the HNE adducts was also seen in some of the control animals fed the high-fat diet (C), whereas not in animals with no treatment (D). (A) Animal no ethanol-1. (B) Animal no ethanol-2. (C) Animal no high-fat diet-1. (D) Animal no ethanol-1. Sites of positive staining are indicated by arrows. Original magnifications, × 250.

0.962, $p < 0.001$) adducts. Although the correlation between the histological score and adduct or cytochrome immunochemistry, except for CYP3A ($r = 0.741$, $p = 0.05$) did not reach significance (Table 2), a significant co-occurrence was found between hepatic steatosis and cytochrome enzymes (Fig. 6).

DISCUSSION

The present data demonstrate that, in the early phase of ethanol-induced liver injury, reactive aldehydic products resulting from ethanol metabolism and lipid peroxidation are formed in the liver, together with increased expression of CYP2E1 and CYP3A. The heterogeneous, yet similar, distribution of both the aldehydic products and the cytochrome enzymes in the centrilobular region of the liver may have important implications for the liver toxicity of ethanol. The appearance of aldehyde adducts in zone 3 hepatocytes is accompanied by increased serum aminotransferase levels, accumulation of fat, and signs of hepatocellular necrosis and inflammation, which is in accordance with previous observations from a micropig model of alcohol consumption.[13,28] It is notable that the first typical histological lesions of alcohol-induced liver injury in man also develop in the centrilobular region.[29]

Cytochrome P-450 family of proteins are known to be of critical importance in the oxidative, peroxidative, and reductive metabolism of numerous xenobiotics.[30] Chronic alcohol consumption is known to lead to an increase in the content of ethanol-inducible CYP2E1 in the liver and to
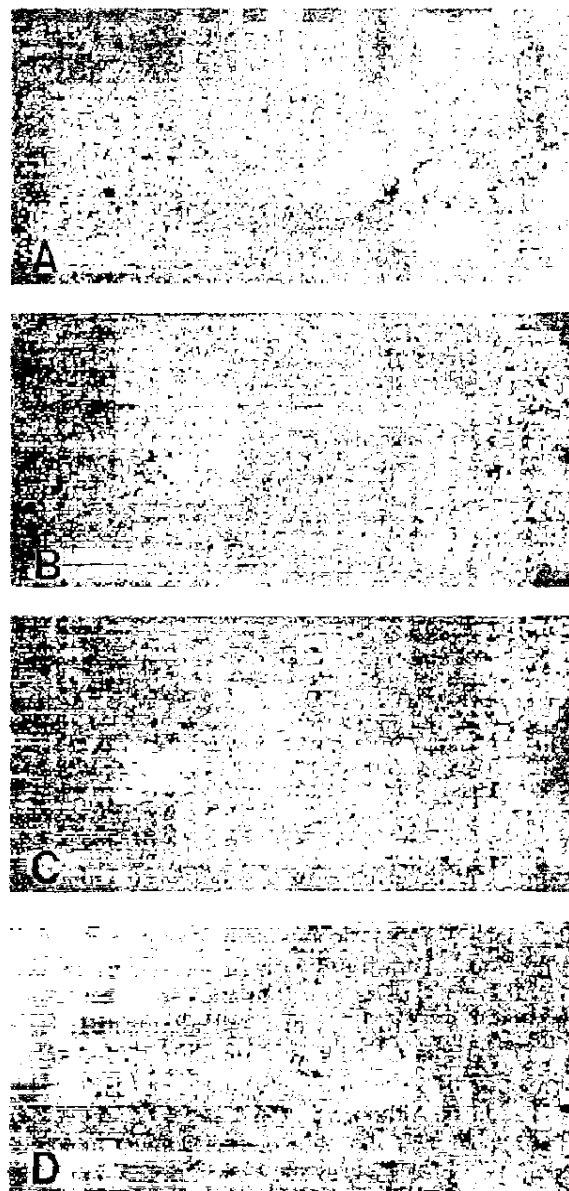

Fig. 4. Control stainings with the nonimmune serum revealed no specific staining in the livers from the alcohol-fed animals (A, B) or from the control animals with (C) or without (D) the high-fat diet. (A) Animal no ethanol-1. (B) Animal no ethanol-2. (C) Animal no high-fat diet-1. (D) Animal no ethanol-1. Original magnifications, × 250.

enhance its catalytic activity in the microsomal fraction.[16-18,31] Induction of CYP2E1 in alcohol-fed animals has been shown to increase the sensitivity of hepatic microsomes to lipid peroxidation.[16] On the other hand, inhibition of CYP2E1 activity may lead to clinical improvement in the early phase of alcoholic liver injury.[17] CYP2E1 metabolizes ethanol via oxidation to the cytotoxin AA and is capable of generating membrane damaging free radicals in the process.[30] Interestingly, the present data also shows abundant amounts of CYP3A enzyme in the alcohol-fed animals. This supports preliminary cell culture findings,

Fig. 5. Immunohistochemical stainings for the cytochrome P-450IIE1 enzyme revealed positive staining in zone 3 after ethanol treatment (A, B). In four ethanol-fed animals and in all of the control animals, the stainings for CYP2E1 remained negative (C). Significant positive correlations emerged between CYP2E1 and MDA ($r = 0.827$, $p < 0.05$) and HNE ($r = 0.884$, $p < 0.01$). (A) Animal no ethanol-1. (B) Animal no ethanol-2. (C) Animal no high-fat diet-1. Original magnifications, × 250.

Fig. 6. CYP3A enzyme was demonstrated in both the control livers and in the ethanol-fed animals. However, the stainings intensities for CYP3A in the centrilobular region were markedly higher in the ethanol-fed animals (A, B) than in the controls (C). CYP3A correlated with AA ($r = 0.810$, $p < 0.05$), MDA (0.888, $p < 0.01$), and HNE ($r = 0.962$, $p < 0.001$) adducts. (A) Animal no ethanol-1. (B) Animal no ethanol-2. (C) Animal no high-fat diet-1. Original magnifications, × 250.

indicating the occurrence of a generalized induction of cytochromes by ethanol consumption.[20,32] The CYP3A subfamily, which is a major component of human P-450s, has been previously known primarily for its role in the activation of other types of hepatotoxins and carcinogens, such as acetaminophen benzo(a)pyrene and aflatoxinB1. Herein, both CYP2E1 and CYP3A were found to be induced and accompanied by formation of AA and lipid peroxidation-derived adducts. Although CYP2E1 and CYP3A expression and the appearance of protein adducts appear to co-occur, it should be noted that, in some alcohol-fed animals, the stainings for CYP2E1 remained negative. Interestingly, recent studies have also emphasized the role of Kupffer cells instead of CYP2E1 in the initiation of hepatocyte damage caused by alcohol.[33] Nevertheless, the present data suggest

that AA formation and enhanced oxidant stress are involved in the initiation of alcohol-induced tissue injury.

Several harmful consequences of protein adduct formation have been postulated, such as interference with protein function[34,35] or stimulation of immunological tissue damage.[36,37] Not surprisingly, adduct formation with CYP2E1 enzyme itself also seem to occur in vivo.[38,39] Whether such reactions could lead to impaired enzymatic function of this cytochrome, too, remains to be established. The generation of reactive aldehydes can also stimulate fibrogenesis and collagen gene expression in cultured fibroblasts and Ito cells.[19,40-42] On the other hand, development of cirrhosis has previously been shown to correlate with the degree of steatosis.[43] Therefore, it is of interest to note that diets high in polyunsaturated fat and low in carbohydrate may induce cytochrome P-4502E1 (CYP2E1)[44] and enhance oxidative stress in the presence of ethanol.[7,45] Apparently, the degree of the oxidant stress and cytochrome P-450 enzyme expression may also be modulated by the high-fat diet alone.[44,45]

To conclude, the present data demonstrates a joint occurrence of AA, and lipid peroxidation-derived protein adducts and enhanced expression of CYP2E1 and CYP3A in the centrilobular region of the liver in an early phase of liver disease. It remains to be established whether such findings may also be implicated in human alcohol abusers frequently consuming excessive amounts of ethanol together with a high-fat diet.

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Table 2. Correlations (Spearman's Rank Correlation Coefficients) Between the Clinical, Histological, and Immunohistochemical Data in the Individual Animals After Ethanol Treatment

	Duration of treatment	Urine alcohol	AST	Histological score	AA	MDA	HNE	CYP2E1
CYP3A	-0.505^{NS}	-0.109^{NS}	0.291^{NS}	0.741^{**}	0.810^{*}	0.888^{**}	0.962^{***}	0.879^{**}
CYP2E1	-0.167^{NS}	-0.297^{NS}	-0.119^{NS}	0.576^{NS}	0.719^{**}	0.827^{*}	0.884^{**}	—
HNE	-0.463^{NS}	-0.283^{NS}	0.226^{NS}	0.606^{NS}	0.901^{**}	0.883^{**}	—	—
MDA	-0.214^{NS}	-0.330^{NS}	0.385^{NS}	0.393^{NS}	0.577^{NS}	—	—	—
AA	-0.729^{**}	-0.336^{NS}	0.168^{NS}	0.629^{NS}	—	—	—	—
Histological score	-0.566^{NS}	0.436^{NS}	-0.055^{NS}	—	—	—	—	—
AST	-0.535^{NS}	$^{1)}0.095^{NS}$†	—	—	—	—	—	—
Urine alcohol	-0.020^{NS}	—	—	—	—	—	—	—

NS, not significant. p values are as follows: $^* p < 0.05$; $^{**} p < 0.01$; $^{***} p < 0.001$.
† Correlation coefficient for linear regression.

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